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16. ABSTRACT

Inoculation of pentachlorophenol-contaminated natural waters with cells of a pentachlorophenol-degrading Flavobacterium was shown to be an effective method for decontamination of PCB-polluted aquatic environments. Numerous types of waters were decontaminated, including: river water, lake water, and groundwater. Decontamination was most effective between 15 C and 30 C, and between pH 7.5 and pH 9.0. Inoculation of waters with as few as 10^4 cells/ml resulted in effective PCP removal. PCB concentrations between 10 ppb and 100 ppm were reduced to undetectable levels, usually within 48 hours. Microbiological decontamination of PCP-polluted waters appears to be a promising waste treatment alternative when compared to traditional treatment techniques.

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MICROBIOLOGICAL DECONTAMINATION OF PENTACHLOROPHENOL-CONTAMINATED NATURAL WATERS

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Summary.

Inoculation of pentachlorophenol-contaminted natural waters with cells of a pentachlorophenol-degrading Flavobacterium was shown to be an effective method for decontamination of PCP-polluted aquatic environments. Numerous types of waters were decontaminated, including: river water, lake water, and groundwater. Decontamination was most effective between 15 C and 30 C, and between pH 7.5 and pH 9.0. Inoculation of waters with as few as 10^4 cells/ml resulted in effective PCP removal. PCP concentrations between 10ppb and 100ppm were reduced to undetectable levels, usually within 48 hours. Microbiological decontamination of PCP-polluted waters appears to be a promising waste treatment alternative when compared to traditional treatment technologies.

INTRODUCTION

Pentachlorophenol (PCP) is a widely used biocide that contaminates soil and water in many parts of the United States (Edgehill and Finn, 1983; Pignatello, et al., 1983). This compound is acutely toxic, so environmental contamination is of concern both as an ecological threat and as a public health menace. It is possible to isolate pure cultures of bacteria that grow on PCP as a sole source of carbon and energy (Chu and Kirsch, 1972; Edgehill and Finn, 1983; Pignatello, et al., 1983; Stanlake and Finn, 1982; Suzuki, 1977; Trevors, 1982; and Watanabe, 1973). Such pure cultures have potential for use as PCP detoxification agents. For example, work by Edgehill and Finn (1983) suggests that inoculation of PCP-contaminated soils with PCP-degrading bacteria may be a feasible method for removing PCP from such environments. Here we show that addition of cells of a PCP-degrading Flavobacterium to PCP-contaminated natural waters is an effective decontamination technique.

MATERIALS AND METHODS

During previous work (Pignatello, et al., 1983) we isolated from PCP-contaminated stream sediments a pure culture of a Gram-negative bacterium that grows aerobically on PCP as a sole source of carbon and energy. This bacterium will tolerate PCP at concentrations of 300-400 mg/L, completely degrading such quantities of PCP within 24-48 hours and liberating all PCP chlorine as chloride ions. Extensive characterization of this strain using the methods of Holmes, et al. (1981), Weeks (1974), Smibert and Krieg (1981), Hendrie and Shewan (1979), Kodaka, et al. (1982), and Skerman (1969), summarized in the RESULTS section, indicates that it belongs in the genus Flavobacterium. We have used this strain for the detoxification studies reported here. The Flavobacterium was grown axenically to mid-logarithmic stage in a defined growth medium (Pignatello, et al., 1983) containing L-glutamic acid (4.0 g/L) as the only source of carbon and energy. Pentachlorophenol then was added (20 ppm) to induce the PCP degradative enzymes. When degradation of the added PCP was n early complete, as determined by measuring ultraviolet absorbance of the culture fluid at 318-320nm (Pignatello, et al., 1983), the microbial cells were added directly (without removal of the spent growth medium) to PCP-contaminated waters to give 106 to 107 cells/ml of water. Cell densities in the initial inoculae were determined by direct microscopic counts (Hobbie, et al., 1977) or by using a standard curve of culture turbidity (A_{550}) vs. cell number/ml.

Concentrations of PCP in natural waters were determined by a gas-chromatographic procedure described previously (Pignatello, et al., 1983), or by measuring the absorbance of water at 318nm (Pignatello, et al., 1983). The latter procedure was used for most samples. The gas-chromatographic procedure was employed when increased sensitivity was needed (<100 ppb) and periodically as a check on the spectrophotometric assay.

Mississippi River water was collected on May 7, 1983 (water temperature, 20 C; oxygen concentration, 8mg/L; pH, 8.2) from outdoor experimental streams at the Monticello Ecological Research Station, a field station of the United States Environmental Protection Agency Environmental Research Laboratory at Duluth, Minnesota (Arthur, et al., 1982). River water (20-liter portions) was distributed into all-glass, 5-gallon aquaria in four treatment groups. Group A (2 replicates) received only water. Group B (2 replicates) received water plus 10^6 bacterial cells/ml. Group C (4 replicates) received water plus 1070-1140 ppb of PCP

(average for the four aquaria, 1110 ppb). Group D (4 replicates) received water, 10^6 bacteria/ml, and PCP (1020-1110 ppb, average = 1080 ppb). Concentrations of PCP within all aquaria were monitored by periodic sampling of the water and determination of [PCP] gas-chromatographically. All aquaria were protected from exposure to direct light to minimize photolytic decomposition of PCP. After 48 hours, 10 fathead minnows (Pimephales promelas) were added to each aquarium, and fish survival monitored up to 168 hours past the beginning of the experiment.

Numerous other water samples were collected from the vacinity of the Gray Freshwater Biological Institute during May and June of 1984. Groundwaters were collected from a well in Deephaven, MN (water pH, 6.9) and a well in Spring Park, MN (pH, 6.4). Oligotrophic lake water (pH, 7.2) was collected from Christmas Lake, near Excelsior, MN. Eutrophic lake water (pH, 6.9) came from Lake Minnetonka, Minnetonka Beach, MN . A second river water sample (pH, 7.1) came from the Crow River near Delano, MN. Portions (100 ml) of each sample were placed in seperate 250ml flasks, and PCP was added to a concentration of 100 ppm. Each flask then received 10^7 Flavobacterium cells/ml. Control flasks received only PCP. All flasks were incubated with shaking, in the dark, at 25 C. Pentachlorophenol concentrations in the various waters were determined periodically by measuring the absorbance of small aliquots of water at 318nm. If necessary, aliquots were centrifuged to remove microbial cells prior to analysis for PCP. Periodic checks of PCP concentration in non-centrifuged aliquots confirmed that decreases in A_{318} were due to disappearance of PCP, not adsorption of PCP to microbial cells.

For examination of effects of pH, temperature, PCP concentration, and Flavobacterium cell density on rates of PCP removal from natural waters, we used water collected from Lake Minnetonka, MN. Freshly-collected water (pH 6.9-7.0) was distributed into flasks (100ml/flask) and supplemented with PCP. For temperature experiments, all flasks received 100ppm of PCP plus approximately 10^7 bacterial cells/ml. They were incubated in the dark, without agitation at temperatures between 15 C and 40 C. To examine effects of Flavobacterium cell density, water samples containing 100ppm of PCP were inoculated with different concentrations of bacterial cells, varying between 5.5×10^4 cells/ml and 5.5×10^7 cells/ml. For pH studies, water samples containing 100ppm of PCP were adjusted to various pH's by addition of bufferring agents (TRIS: pH's 8.0-9.0, MES: pH's 6.0-7.0, phosphoric acid: pH's 7.0-8.0, and borate: pH's 9.0-10.0) at a concentration of 10mM. At transition pH's (e.g., pH 7.5) duplicates were run with different bufferring agents (e.g., TRIS and phosphoric acid)

to confirm that effects on PCP removal rates were due to pH and not particular types of bufferring agents. After adjustment of pH, water samples were inoculated with approximately 10^7 Flavobacterium cells/ml; pH's were monitored throughout the experiment, and maintained within 0.1 unit of the initial pH. To examine the effects of PCP concentration on removal rates, two approaches were used. In the first, a moderately low PCP concentration of 1.0ppm was used, with variable densities of the Flavobacterium. In the second, all flasks received the same density of bacteria (10^7 cells/ml), but different concentrations of PCP (0.1, 1.0, 10.0, and 100.0ppm). Flasks for pH, inoculae size, and PCP concentration studies were incubated in the dark, without agitation, at 25 C. Aliquots of water were removed periodically for determination of [PCP].

RESULTS AND DISCUSSION

We have isolated from a variety of PCP-contaminated environments about 40 pure cultures of bacteria that are able to use PCP as a sole source of caron and energy for growth. All of these strains are Gram-negative and exhibit a yellow insoluble pigment. They grow optimally between 25 and 30 C, and tolerate PCP at concentrations up to 300-400ppm. A single strain was used in the detoxification studies reported here. This bacterium is rod-shaped, about 2 micometers long. It is not motile. It possesses cytoplasmic inclusions when grown on rich media, and exhibits positive tests for oxidase, catalase, and phosphatase. It gave negative tests with respect to the following: casein hydrolysis, gelatin hydrolysis, lipase, amylase, arginine dihydrolase, N_2 production from nitrate, indole production, urea hydrolysis, MRVP test, modifications of litmus milk, deoxyribonuclease production, growth on nitrogen-free media, growth on MacConkey Agar, cellulase production, agar degradation, and growth at 40 C. The bacterium will grow in the presence of 2% NaCl, and produces ammonia when grown on peptone. It grows well microaerophilically, and in the presence of low oxygen concentrations produces acid from glucose, maltose, trehalose, salicin, and cellobiose. Under strictly anaerobic conditions it will not ferment glucose. No acid is produced under aerobic conditions from zylose, mannose, fructose, maltose, sorbose, galactose, salicin, inulin, dextrin, pyruvate, acetate, succinate, aspartate, glutamate, or butyrate. The bacterium will not grow at the expense of sucrose, lactose, rhamnose, mannitol, raffinose, arabinose, dulcitol, inositol, glucosamine, gluconate, tartarate, β-alanine, propionate, salicylate, valerate, oxalate, ethanol, propanol, citrate, trehalose, benzoate, glycine, fumarate, arginine, starch, 2-chlorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate, 2,4-dichlorobenzoate, 4,6-dichloro-resorcinol, o-chlorophenol, m-chlorophenol, p-chlorophenol, 2,4-dichlorophenol, or 2,4-dichlorophenoxyacetic acid when these compounds are supplied as the only source of carbon and energy at a concentration of 100ppm in a defined mineral salts medium. A light to heavy pellicle developes upon stationary growth in liquid media. The bacterium is resistant to Novobiacin at a disc concentration of 30mcg. The microorganism grows at the expense of PCP, acetate, succinate,glucose, maltose, mannose, cellobiose, galactose,salicin, inulin, dextrin, pyruvate, aspartate, butyrate, glutamate, and β-hydroxybutyrate when these are supplied as sole sources of carbon. Good growth occurs between pH 6.9 and pH 8.5, and at temperatures between 15 C and 37 C. No fluorescent pigments are produced. The guanine + cytosine content of the bacterium's DNA is 63%.

The bacterium harbors one or more plasmids. The above characteristics indicate that this bacterium should be classified in the genus Flavobacterium (Holmes, et al., 1984; Smibert and Krieg, 1981; Hendrie and Shewan, 1979; and Kodaka, et al., 1982).

Mississippi River water was distributed into aquaria as described under MATERIALS AND METHODS. All aquaria, except treatment group A, received approximately 1ppm of PCP. Some aquaria also received bacteria, while others did not. Addition of approximately 10 Flavobacterium cells/ml to PCP-contaminated river water resulted in removal of >90% of the biocide within 48 hours, as summarized in Table 1. Removal of the PCP detoxified the water, as shown by survival of PCP-sensitive minnows in treated water, but not in untreated water (Table 2).

The ability of the Flavobacterium to remove PCP from natural waters other than that from the Mississippi River was examined. The bacterium readily decontaminated five additional waters, including oligotrophic (low productivity) lake water, eutrophic (highly productive) lake water, groundwater, and another river water. Four of the water samples were decontaminated of 100ppm of PCP within about 48 hours, at which time PCP no longer was detectable. One well water sample (Spring Park, MN) was decontaminated completely, but only after a 72-hour lag period. We suspect this was due to the lower initial pH of this water as compared to the others. Other experiments (see below) indicated that pH's of <7.0 inhibited PCP removal by the Flavobacterium.

Temperature was an important variable affecting PCP removal rates. The Flavobacterium removed PCP within 50 hours at between 20 C and 30 C. Removal rates slowed somewhat at 15 C (~110 hours for complete removal), but still were significant. In most experiments, slow removal of PCP was observed 35 C, with no removal at 40 C. In general it appears that temperatures in excess of about 35 C are detrimental to PCP removal. Shifting of a water sample between 25 C and 35 C at 10-14 hour intervals, as might occur in an outdoor treatment system due to diurnal variations of temperature, only slightly slowed PCP removal as compared to incubations at 25 C or 30 C without variation. Thus, exposure of the bacterium to 35 C for periods of 10-14 hours did not inactivate the cells. This observation is encouraging as regards outdoor applications in warm climates.

Inoculum density also affected PCP removal rates. Increasing the densities of Flavobacterium cells significantly increased PCP removal rates, as shown in Figure 1. However, even rather low densities of the

bacterium (e.g., 5.5×10^4 cells/ml) were reasonably effective at removal of PCP.

The pH optimum for removal of PCP from lake water was about pH 8.5. Rapid removal of PCP occurred between pH 7.5 and pH 9.0. The Flavobacterium still was active at pH's as low as pH 6.5, but removal rates slowed considerably at these pH's. No removal was observed at pH 6.0. Thus, the bacterium performs best as a PCP decontamination agent at somewhat alkaline pH's. It performs poorly at pH 7.0 or lower, or pH 9.5 and higher. Rates of PCP removal varied slightly depending on the type of water being decontaminated and in water samples collected at different times of the year; however, the overall response to pH (optimum, pH ~8.5) was consistent.

The Flavobacterium was able to remove PCP from natural waters over a wide range of PCP concentrations. PCP concentrations ranging between 10ppb and 100ppm were decontaminated equally well. Rates of PCP removal (% PCP removed/hour) were similar at all concentrations, but total time required for complete removal increased with increasing PCP concentration because of somewhat longer initial lag phases preceding degradation at the higher PCP concentrations. PCP concentrations usually were reduced to levels below our detection limit of about 0.1 ppb.

Decontamination of natural waters that have been polluted with PCP clearly may be accomplished using microbiological techniques. As with any biological system, many variables may influence the outcome of decontamination attempts. Some particularly important variables include pH, temperature, pollutant concentration, type of natural water to be treated, density of added microorganisms, and the abilities of the added microbes to compete with the natural microflora already in the water. Data reported here show that our PCP-degrading Flavobacterium is a very versatile PCP decontamination agent. It removes PCP from most types of natural water. It accomplishes this quite rapidly and over a range of pH and temperature that encompasses conditions encountered in most natural systems. Either high or low concentrations of PCP are treatable, and PCP concentrations in treated water are reduced to very safe levels, usually below detection by gas-chromatographic measurement techniques. As few as 10^4-10^5 Flavobacterium cells/ml are sufficient to detoxify most waters. Ongoing experiments indicate that the costs of growing the Flavobacterium, concentrating the cells, and transporting them to a pollution site will be competitive with existing clean-up technologies such as the use of activated carbon filtration. Results of several large field trials of our microbiological clean-up process will be reported later.

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Table 1. Removal of Pentachlorophenol from River Water by a Flavobacterium

TIME (hours)		% ORIGINAL PCP REMAINING	
		Aquaria with Bacteria (Treatment Group D)	Uninoculated Aquaria (Treatment Group C)
0		100.0 ±4.0	100.0 ±2.5
4		93.8 ±4.0	103.2 ±1.2
8		87.0 ±2.9	102.8 ±0.5
12		79.2 ±7.9	98.3 ±0.9
20		64.2 ±9.7	100.5 ±7.0
34		40.3 ±14.9	102.0 ±9.2
45		24.5 ±15.6	105.5 ±15.3
48	(fish added)	ND	ND
51		17.4 ±12.6	92.1 ±0.5
57		11.7 ±9.9	94.4 ±4.8
69		9.3 ±8.9	89.7 ±3.8

ND, not determined
Results are ± standard deviation (Young, 1962)

Table 2. Fish Survival in Contaminated and Decontaminated River Water

TREATMENT GROUP	% FISH SURVIVAL (Hours, following addition of fish)										
	0.0	2.0	2.5	3.0	3.5	4.0	6.0	9.0	10	48	168
A	100	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100	100
C	100	90	48	12	8	0	0	0	0	0	0
D	100	100	100	100	100	95	90	85	80	78	78

A, no bacteria/no PCP; B, bacteria/no PCP; C, PCP/no bacteria; D, PCP and bacteria. The difference between treatment groups C and D after 168 hours is significant at the 95% level (Young, 1962). Of the fish that did not survive in treatment group D, all were in a single, atypical aquarium; no fish were lost in 3 of the 4 replicate aquaria.

Figure 1. Effect of Inoculum Size on Removal of Pentachlorophenol from Natural Water by a Flavobacterium

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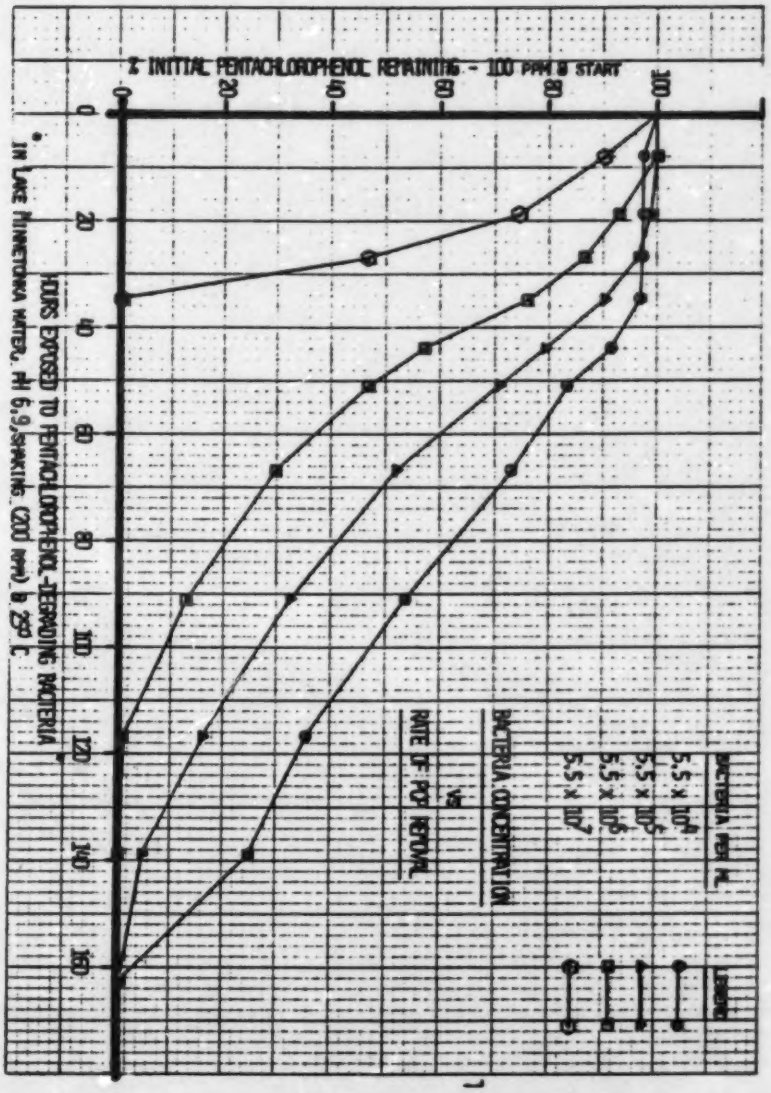
% INITIAL PENTACHLOROPHENOL REMAINING - 100 PPM @ START
0
20
40
60
80
100
120
140
160
HOURS EXPOSED TO PENTACHLOROPHENOL-DEGRADING BACTERIA
* IN LAKE MINNETONKA WATER, pH 6.9, SPIKING COD (PPM) @ 25° C.
BACTERIA PER ML
5.5 x 10^4
5.5 x 10^5
5.5 x 10^6
5.5 x 10^7
BACTERIA CONCENTRATION
VS
RATE OF POP REMOVAL